Predicting Response to Lisinopril in Treating Hypertension: A Pilot Study

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Supplemental Methods

Study design: This study is a secondary analysis of a parent trial examining the pharmacogenomic effectiveness of metoprolol succinate in HTN patients (NCT02293096). For the parent trial, patients between 18 and 89 years of age who had uncontrolled HTN, defined as >140/90mmHg, were enrolled from patients presenting to the emergency department at the University of Colorado Anschutz Medical Campus as well as well as those referred from clinics in the Denver, Colorado and surrounding area communities. Patients that were pregnant, had organ failure, illicit drug use, or allergy to metoprolol succinate were excluded from the study. Patients were advised not to drink coffee or take other stimulants prior to the study visit. At the initial study visit, baseline blood pressure and plasma samples (prepared from whole blood collected in K2 EDTA anticoagulated tubes (Lawson #12016) and stored at -80°C) were collected prior to receiving lisinopril therapy (10 mg daily). Blood pressure was reevaluated at the next study visit (~1 week later) and the same method was used for blood pressure assessment at each visit. These were conducted using an automated oscillometric inflatable cuff after 10 minutes seated in a quiet room with a research assistant. Blood pressured were taken three times and the average of these three for each visit were recorded. Patients were defined as “responders” to lisinopril therapy if they achieved a 10% decline in SBP between visits. All others were defined as “non-responders”. Clinical parameters (race, ethnicity, age, sex, body mass index, systolic blood pressure, diastolic blood pressure, heart rate, creatinine level, calculated estimated glomerular filtration rate (GFR), diabetic status, smoking status, comprehensive list of all medications currently taken including other antihypertensive medications, date and time of when each medication was last taken – including lisinopril) and adverse drug events (as outlined in the U.S. Food and Drug Administration, FDA 21 312.32 Code of Federal Regulations) were also collected at each visit. GFR was calculated using the Cockcroft Gault equation. All subjects reported compliance to taking lisinopril at follow-up. Subjects also brought in their current medications to verify which they were taking. For each

medication the subject was on, the medication name, dose, route and date/time were documented. Dietary records were not included. Prior to our analysis, we reviewed in detail each of the visit instruments including the summary notes and excluded subjects that were no longer taking lisinopril, were not compliant to study protocol, were already on lisinopril prior to starting the study, and subjects whose samples were stored incorrectly prior to analysis.

Metabolite extraction and identification: We compared provocative plasma samples from the baseline visit. Samples were prepared for UHPLC-MS metabolomics in the following manner. Polar metabolites were extracted from plasma (20 µL) in ice-cold lysis/extraction buffer (methanol:acetonitrile:water 5:3:2) at a 1:25 dilution. Prior to extraction, isotopically labeled standards (See Supplemental Table 1) were added at expected biological concentrations to the lysis buffer for absolute quantitation. Samples were agitated (30 min, 4°C) followed by centrifugation (18,213 g, 10 min, 4 °C). Protein pellets were discarded, and supernatants were stored at -80°C prior to metabolomic analyses. Plasma extracts were injected (20 µL) into a Thermo Vanquish UHPLC system (San Jose, CA, USA) coupled to a Thermo Q Exactive mass spectrometer with electrospray ionization (Bremen, Germany). Metabolites were separated on a Kinetex C18 column (150 x 2.1 mm, 1.7 µm – Phenomenex, Torrance, CA, USA) at 45°C using a five-minute gradient method at 450 µl/min and mobile phases (A: water/0.1% formic acid; B: acetonitrile/0.1% formic acid) for positive ion mode. Solvent gradient: 0-0.5 min 5% B; 0.5-1.1 min 5-95% B, 1.1-2.75 min hold at 95% B, 2.75-3 min 95-5% B, 3-5 min hold at 5% B. Negative ion mode used the same five minute gradient method at 450 µl/min, with the following changes: 1 mM ammonium acetate (NH_4OAc) substituted for 0.1% formic acid (A: 95/5 water/acetonitrile 1 mM NH_4OAc; B: 5/95 water/acetonitrile 1 mM NH_4OAc). Solvent gradient: 0-0.5 min 0% B; 0.5-1.1 min 0-100% B, 1.1-2.75 min hold at 100% B, 2.75-3 min 100-0% B, 3-5 min hold at 0% B. Non-polar metabolites were extracted from plasma (20 µL) in ice-cold methanol at a 1:10 dilution. Samples were quickly hand vortexed at room temperature followed by incubation at -

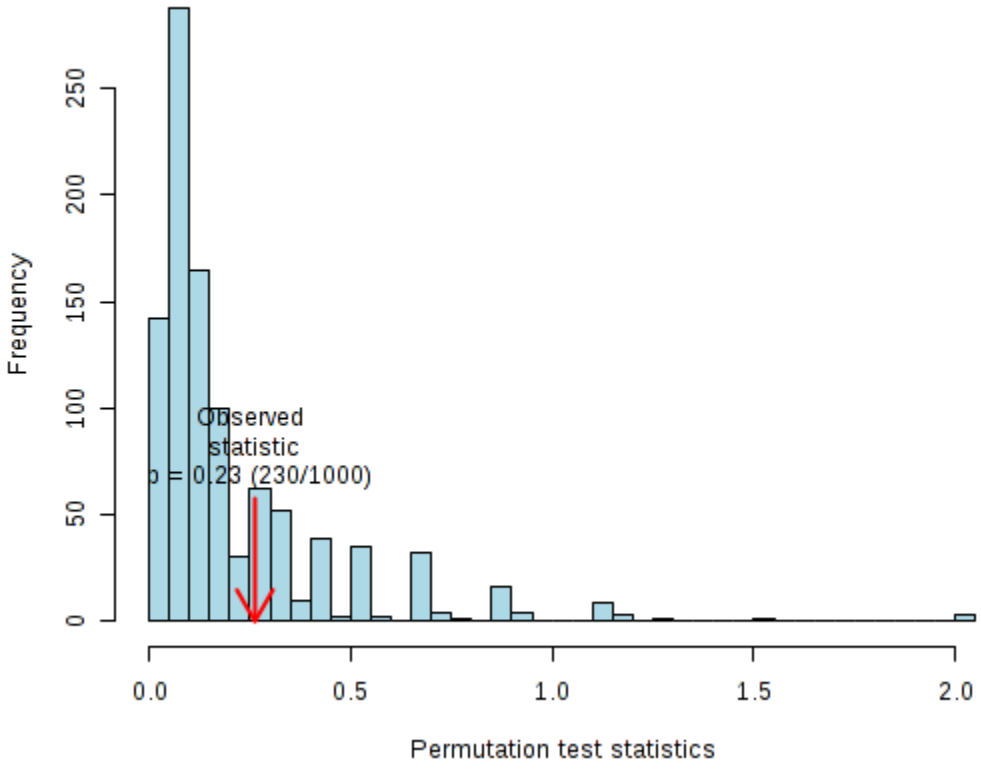
20°C for 30 minutes. Samples were centrifuged (18,213 g, 10 min, 4 °C) and supernatants were diluted 1:1 using 10 mM ammonium acetate. Protein pellets were discarded, and diluted supernatants were stored at -80°C prior to metabolomic analyses. Diluted plasma extracts were injected (10 µL) into a Thermo Vanquish UHPLC system (San Jose, CA, USA) coupled to a Thermo Q Exactive mass spectrometer with electrospray ionization (Bremen, Germany). Metabolites were separated on an Acquity HSS T3 column (150 x 2.1 mm, 1.8 µm – Waters, Milford, MA, USA) at 45°C using a seventeen minute gradient method at 300 µl/min (unless where noted) and mobile phases containing IPA (isopropanol, A: 72:25 H2O:ACN 5 mM NH_4OAc, B: 50:45:5 IPA:ACN:H2O 5 mM NH_4OAc) for negative ion mode. Solvent gradient: 0-1.0 min 25% B; 1.0-2.0 min 50% B; 2.0-8.0 min 90% B; 8.0-10.0 min 99% B; 10.0 – 14.0 min hold at 99% B; 14.0-14.1 min 25% B, 400 µL/min; 14.1-16.9 min hold at 25% B, 400 µL/min; 16.9-17.0 min hold at 25% B. For each method, quality controls were generated from pooled aliquots of extracts, and were run every 15 analytical runs, to control for technical variability, as judged by coefficients of variation (CV). CV were determined by calculating the ratios of standard deviations divided by mean measurements for compounds of interest across all technical mix runs. Data files were converted to .mzXML format followed by being analyzed in Maven (Princeton, NJ, USA) for metabolite identification. During the metabolite identification process, quality controls were initially assessed to make sure that they appeared relatively identical to verify adequate mixing of the aliquots. Blank samples were observed to ensure that they did not express metabolite levels. Each metabolite was identified by its mass (observed for a distinct and pronounced peak across samples at designated mass) and confirming with an associated C13 peak. Metabolites whose peak intensities were comparable to background levels in samples investigated were not included for analysis. The metabolomics and metadata reported in this paper are available via study ID: MTBLS1021 at https://www.ebi.ac.uk/metabolights/

Identification of statistically significant variables: For all clinical and demographic variables, chi-square (for categorical variables) or t tests (for continuous variables) were performed to identify which produced statistically significant results while concurrently using a Bonferroni multiple comparisons correction on these analyses. Separately, t tests were performed on the relative quantitation values for the individual metabolites identified as being present in the investigated plasma samples. A Bonferroni multiple comparisons correction was applied to these results. Additionally, fold change differences between expression of the metabolites in relative quantitation between the responder and non-responder groups. Logistic regression modeling was then used to identify which metabolites and clinical variables produced the best model. Backwards selection based on p-value for the individual variables in conjunction with model fitting significance, Pearson goodness of fit significance and McFadden pseudo R-square values. Absolute quantitation results for 2-oxoglutarate were obtained from the same cohort to validate the results from the analyses completed on the relative quantitation results. The results of the power analysis describe the need for a larger validation cohort. Therefore, there is risk that our results may be showing as significant due to overfitting rather than positively identifying an important correlation and therefore follow-up validation cohorts will need to be adequately powered to reduce this risk.

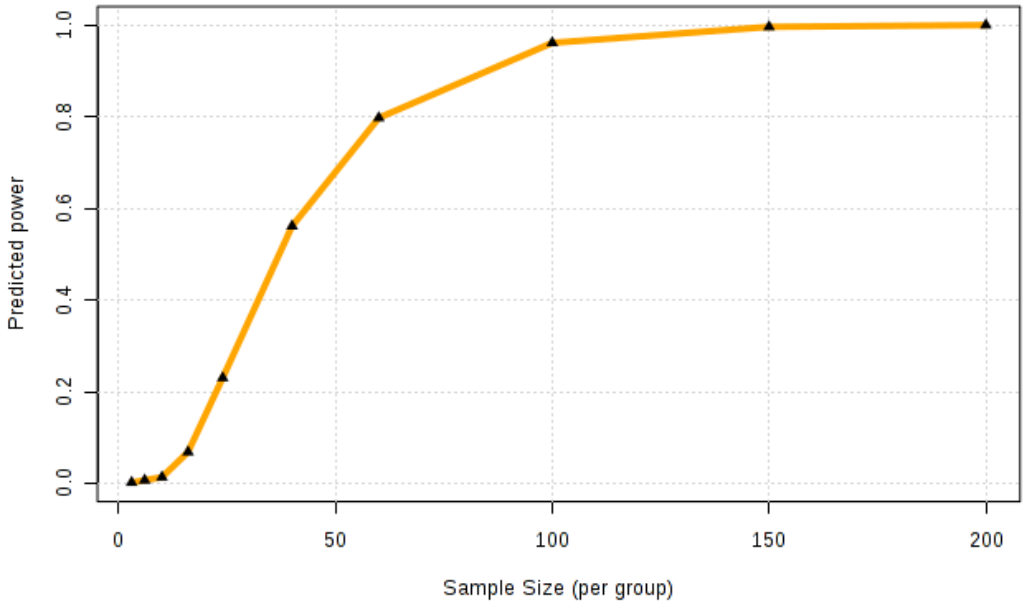
Supplemental Results

Amino Acids	Other
Tyrosine	Palmitate
Valine	Hypoxanthine
Alanine	Citrate
Arginine	Fumarate
Aspartate	D-Glucose
Cystine	2-Oxoglutarate
Glutamate	Lactate
Histidine	Succinate
Lysine	Pyruvate
Methionine	
Phenylalanine	
Proline	
Serine	
Threonine	

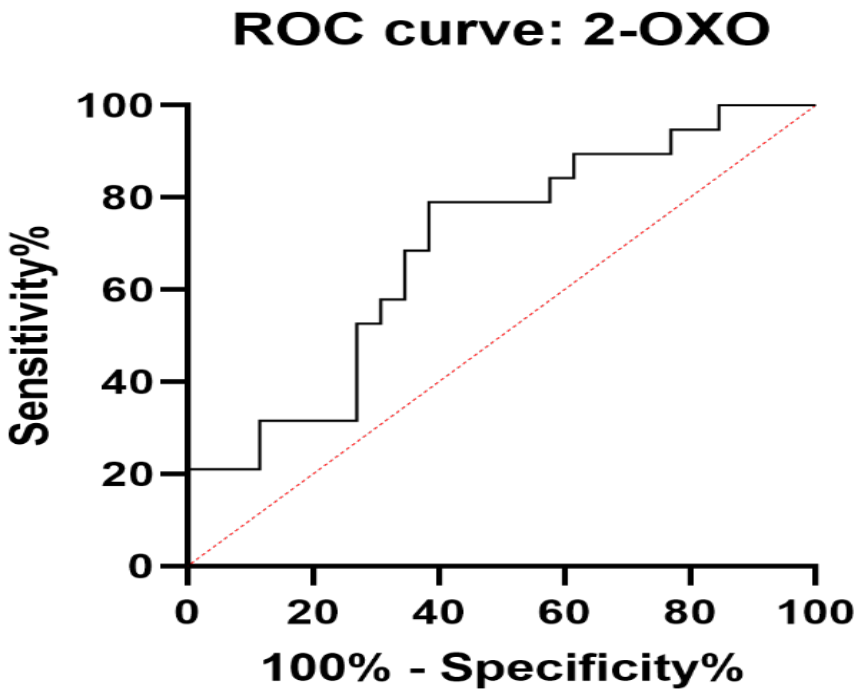
Supplemental Table 1. Metabolites analyzed via absolute quantitation methods: Isotopically labeled standards that were added at expected biological concentrations to the lysis buffer prior to analysis by UHPLC-MS/MS



Supplemental Figure 1. Permutation testing on absolute quantitation metabolites: Permutation testing plot using 1000 permutations run on absolute quantitation data. Area Under Receiver Operating Curve was 0.69, confidence interval of 0.53-0.85, p = 0.03.



Supplemental Figure 2. Power Analysis: Power analysis completed on absolute quantitation data for 2-oxoglutarate. For a power level of 0.8, 60 subjects per treatment group are required for validation cohort.



Supplemental Figure 3. Receiver Operating Curve: Area Under Receiver Operating Curve (AUROC) was 0.69, confidence interval of 0.53-0.85, p = 0.03. This suggests there is a fair level of certainty that 2-oxoglutarate has potential to be a marker predictive of response to lisinopril.